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Efficacy of amylase for wastewater treatment from *Penicillium* sp. SP2 isolated from stagnant water

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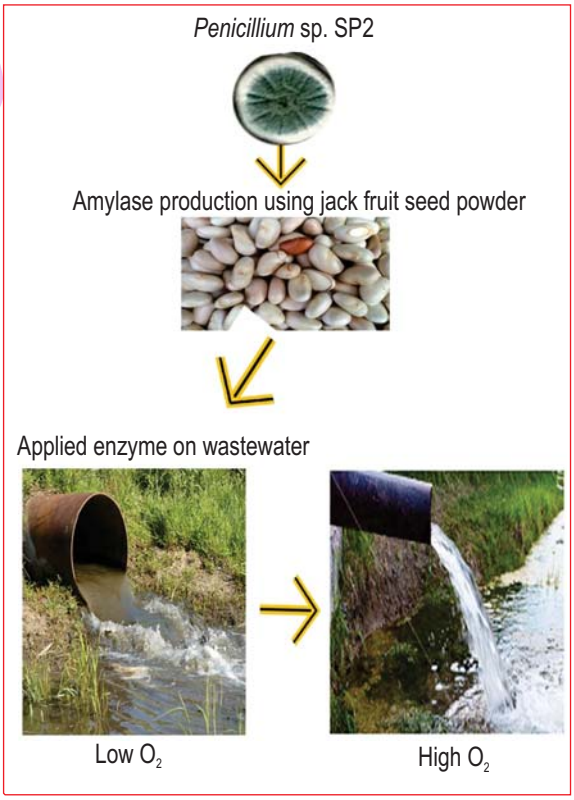
Abstract

Aim : Amylolytic enzymes are useful in bakery, food, automation dishwashing, fodder, wastewater treatment and poultry. The main objective of the present study was to utilize jack fruit seed as a cheap substrate for the production of amylase in solid substrate fermentation using *Penicillium* sp. SP2 and to treat wastewater.

Methodology : Stagnant water was used to isolate *Penicillium* sp. SP2 for amylase production. The potent fungal isolate, *Penicillium* sp. SP2 was cultured in solid substrate fermentation using jack fruit seed as a low cost substrate. The process parameters were optimized to increase the production of enzyme. Amylase was initially fractionated using ammonium sulphate and purified using sephadex G-75 gel filtration column. The crude amylase from *Penicillium* sp. SP2 was applied to treat wastewater.



Results : The isolated *Penicillium* sp. SP2 utilized jack fruit seed substrate and produced amylase. Among the carbon sources, glucose significantly increased the production of enzyme. Beef extract increased amylase production than other nitrogen source. Ca^{2+} ion also significantly enhanced the production of amylase. Amylase production was found to be maximum at 35 °C. The molecular weight of purified amylase was 57 kDa as judged by SDS - PAGE. The crude enzyme reduced carbohydrate content in wastewater and increased dissolved oxygen considerably.

Interpretation : Considering cheap cost, availability, and nutritive value, jack fruit seed is a novel substrate for amylase production for various biotechnological applications. The amylase from *Penicillium* sp. SP2 find great application in wastewater treatment.

Introduction

Amylases (EC 3.2.1.1) are produced by fungi, bacteria and plants (Gupta *et al*., 2003; Wanderley *et al*., 2004). The amylases produced by the fungi are mainly used in brewing, food, baking, distilling, textile and paper industries (Pandey *et al*., 2000). Amylases are classified into α-amylase and β-glucoamylases based on the mode of action. α-amylases act on glucose units and cleave α-1,4 glucosidic linkages between glucose units in the linear amylase chain and glucoamylase readily hydrolyses single glucose units from the non reducing ends of amylase and amylopectin (Sudo *et al*., 1994; Spohr *et al*., 1998). Bacterial amylases have limited specificity and its application is limited in industry. Fungal amylases are mainly preferred to other bacterial sources, mainly due to high accepted generally regarded as safe status (Prakasham *et al*., 2006).

Solid substrate fermentation (SSF) and submerged fermentation (SmF) are mainly used for the production of secondary metabolites, enzymes, and so on. SmF is frequently used for the production of commercially important enzymes (Hashemi *et al*., 2012). SSF is defined as any type of fermentation process that implicates solids or suspensions of solid particles in a liquid phase (Moo-Young *et al*., 1983). In SSF, agro-residues, namely, rice bran, coconut oil cake and wheat bran have replaced the high-cost media mainly used in the SmF for α-amylase production because of their simplicity, low cost, easy availability, better productivity and lesser water output. In addition, it solves the pollution problem occurring due to their disposal in the surrounding. High starch content of almost all the agrowastes (60%–70% by weight) can be effectively used as a major nutrient source by microorganisms such as bacteria, fungi, and so forth for the synthesis of enzymes and these agrowastes also control catabolic repression (Stredansky *et al*., 1999).

Among fungal isolates, many *Penicillium* sp. and *Aspergillus* sp. were frequently used for the production of amylolytic enzymes (Pandey *et al*., 2000). *P. ellutanum* was used recently to produce α-amylase by SmF (Erdal and Taskin, 2010). The organism, such as *P. expansum* MT-1, was used to produce amylases in SSF using as substrate. loquat (*Eriobotrya japonica lindley*) and kernels (Balkan and Ertan, 2007). *P. chrysogenum* was used for producing amylase by SSF using various substrates such as wheat bran, corncob leaf, wheat straw (Goto *et al*., 1998) and tapioca peel powder (Vijayaraghavan *et al*., 2011). The objective of the present investigation was to utilize jack fruit seed powder as a low-cost, novel substrate for the production of amylase using *Penicillium* sp. SP2 in SSF for wastewater treatment.

Materials and Methods

Isolation of fungi for amylase production : Stagnant water was collected aseptically in a plastic container India and transported to the laboratory. from Nagercoil, Tamilnadu, for isolation of fungi. The samples were serially diluted upto 10^{-7} dilution and platted on potato dextrose agar medium [(g l^{-1}) potatoes infusion – 200; dextrose – 20; agar – 15; pH 5.6 ± 0.2] containing streptomycin (0.1 mg ml^{-1}). After that, the plates were incubated at 28 °C ± 2 °C (Johnson and Curl, 1972). The growth was monitored daily, and morphologically different isolates were further purified and subcultured using potato dextrose agar agar medium.

Screening of fungi for amylase production : In the present study, twenty fungal isolates were grown on the starch agar medium (0.1% starch, 0.3% peptone, and 2% agar). The plates were incubated at 37 °C for 72 hrs. Further, 1% (v/v) Lugol's iodine solution (1% iodine and 2% potassium iodide) was flooded in the culture plates. The appearance of blue colour indicated amylase positive (Maria *et al*., 2005; Suganthi *et al*., 2011).

Identification of fungal isolate : Among the twenty fungal isolates, the potent fungal isolate was selected for amylase production and identified on the basis of morphological characters (Barnett and Hunter, 1972). The 18S rRNA ITS region was amplified using MJ Research PTC-225 Peltier Thermal Cycler machine. The forward (5'TCCGTAGGTGAACC-TGCGG3'), reverse primer (5'TCCTCCGCTTATTGATATGC3'), template Deoxyribo-nucleic acid (2 µl) was added to 40 µl of PCR reaction mixture. The reaction conditions were: initial denaturation (94 °C for 2 min) and 35 cycles (94 °C for 45 s, 55 °C for 60 sec and 72 °C for 60 sec) and final extension (72 °C for 10 min). A negative and a positive control were used in the PCR reaction. NCBI BLAST was applied to compare the sequence with the available fungal sequences. The organism was identified as *Penicillium* sp. SP2. The 18S rRNA sequence was submitted to GenBank and accession number was assigned.

Inoculum preparation : The spores of *Penicillium* sp. SP2 was mixed with 10 ml of sterile double distilled water. It was previously cultured for 8 days using PDA slant. To dislodge the spores, an aseptic needle was used and this was used as the inoculum for the production of amylase.

Substrate : Jack bean seeds were procured from a local market at Nagercoil, Kanyakumari district, Tamilnadu. The seeds were dried for 7 days in sunlight, powdered using mixture grinder and used as a substrate for SSF.

SSF for amylase production : SSF was carried out in the Erlenmeyer flasks containing 10% (w/v) jack fruit seed powder in 100-ml double distilled water. The culture medium was sterilized at 121 °C for 20 min and inoculated with inoculums. It was incubated for 8 days at 37 °C and enzyme activity was assayed every 24 hrs.

Enzyme extraction : The fermented culture medium was initially filtered using cotton and was centrifuged (10,000rpm, 20 min) at 4 °C. The supernatant was collected and used as a source of amylase. It was stored at 2 – 8 °C for further studies.

Amylase assay : Alpha amylase assay was carried out using 3, 5-dinitrosalicylic acid method. The reaction mixture contained 0.1

ml crude enzyme and 1 ml soluble starch (1%, w/v) prepared in sodium phosphate buffer (0.1 M, pH = 6.0). The reaction mixture was incubated for 30 min at 37 °C followed by the addition of DNS reagent. The development of colour was measured at 540 nm using a UV-VIS spectrophotometer and the liberated reducing sugar was assayed. A unit of enzyme activity is defined as the amount of enzyme that liberated one µmol of maltose min^{-1} under the assay condition (Miller *et al*., 1959).

Optimization of amylase production : The process parameter for the production of amylase was optimized. The experiment adopted for optimization of factors affecting amylase production was to elucidate the effect of each process parameter independently keeping others bioprocess parameters as constant level. The optimized process parameters were used in subsequent experiments. All experiments were conducted in triplicate and the average values were used. The effect of jack fruit seed on amylase production was evaluated by culturing *Penicillum* sp. in the culture medium at 2%–10% of jack fruit seed. For further studies, 8% jack fruit seed was used as substrate. To evaluate the optimum pH on amylase production, the experiment was performed with varying pH values (4.0, 5.0, 6.0, 7.0 and 8.0) and amylase activity was assayed. To study the effect of temperature on amylase production, the Erlenmayer flasks were incubated at 25 °C, 30 °C, 35 °C, 40°C and 45 °C. To elucidate the effect of inoculum on amylase production, 3-15% inoculum was added. To evaluate the effect of fermentation period on enzyme production, *Penicillium* sp. SP2 was incubated in the fermentation medium and incubated for 1–8 days at 30 °C ± 2 °C. To find the suitable carbon source for enzyme production, different carbon sources, such as glucose, starch, lactose, sucrose and fructose (1%, w/v) were assessed for enzyme production. To evaluate a suitable nitrogen source on enzyme production, the fermentation medium was prepared with urea, casein, yeast extract, peptone and beef extract (1%, w/v). To find the effect of metal ions on enzyme production, jack fruit seed was inoculated with 0.1% metal ions (potassium, iron, sodium, calcium, chloride and magnesium). Tests were conducted in triplicates, and the error bar represents standard deviation.

Purification of enzyme : The crude enzyme was centrifuged (10,000 rpm, 10 min) at 4 °C. To the crude enzyme, ammonium sulphate was added upto 30% saturation and incubated at 4 °C for overnight. The sample was then centrifuged (10,000 rpm for 10 min) at 4 °C and the precipitate was discarded. To the supernatant, ammonium sulphate was added upto 80% saturation, and left at 4 °C for overnight. The precipitate was recovered by centrifugation (10,000 ×g, 10 min) at 4 °C and dissolved in sodium phosphate buffer (0.1 M, pH 7.0) and dialyzed against double distilled water for overnight. Enzyme activity analyzed was purified using sephadex G-75 gel filtration chromatography (Amersham Biosciences, Sweden) (0.6 × 60 cm). The column was equilibrated with 0.1 M sodium phosphate buffer (pH 7.0). The flow rate was adjusted to 1.0 / min and twenty fractions were collected. All fractions were subjected to total protein estimation and amylase activity. The highly active fractions were subjected to sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS – PAGE). 12% (w/v) separating and 5% (w/v) stacking gels were prepared. Equal volume of sample and sample buffer was mixed and kept on boiling water bath for 1 min and loaded (Fincan and Enez, 2014).

Treatment of waste water : The amylase from *Penicillium* sp. SP2 was applied for wastewater treatment studies. The enzyme activity was checked with commercially available amylase. Artificial wastewater was prepared as described earlier (Kapdan and Erten, 2007). To one liter wastewater, 10 mg commercial grade amylase and 10 mg lyophilized crude amylase from *Penicilllium* sp. SP2 were added. The sample mixture was incubated for 1 hr at room temperature (30 ± 2 °C). No enzyme was added to the blank. The dissolved oxygen (DO) content was continuously monitored.

Results and Discussion

Twenty fungi were isolated from water sample. Screening was carried out by starch hydrolysis and out of twenty fungi seven were found to be amylase positive (Table 1). Among the fungal isolates (*Penicillium* sp. 1S, *Penicillium* sp. SP1, *Penicillium* sp. 3S, *Rhizopus* sp. 1, *Rhizopus* sp. 2, *Aspergillus* sp., and *Trichoderma* sp.), *Penicillium* sp. SP2 exhibited potent activity on starch agar medium and was selected for further studies.

Optimization of process parameters and altering the composition of media are one of the typical techniques used for the production of enzymes (Tanyildizi *et al*., 2005). Amylase production from fungi mainly depends on both morphological and metabolic rate of the culture. In case of fungi, growth of mycelium is one of the crucial factors for extracellular amylase production (Carlsen *et al*., 1996a). The physical and chemical factors such as temperature, pH, carbon and nitrogen sources act as inducer in enzyme bioprocess. These process parameters significantly influenced the amylase production (Shivaramkrishnan *et al*., 2006).

In SSF, selection of a suitable cheap culture medium is key factor that involves screening of many solid substrates for the growth and production of enzymes (Kunameni *et al*., 2005). The cheap agro-industrial residues were used as solid

Table 1 : Amylase producing fungi isolated from stagnant water and its activity

Organisms	Zone (mm)
Penicillium sp. IS1	9
Penicillium sp. 3S	2.5
Penicillium sp. SP2	12.5
Rhizopus sp. 1	7
Rhizopus sp. 2	6.5
Aspergillus sp.	8
Trichoderma sp.	11

substrate for the production of ethanol, α – amylase, lactic acid and commercially important enzymes (Huitron *et al*., 2008; Ray *et al*., 2008; Ray et al., 2009). In the present investigation, the jack fruit seed powder was used as solid medium for amylase production. In *Penicillium* sp. SP2, 8% Jack fruit seed powder was optimum for amylase production, after which enzyme production gradually declined (Fig. 1a). Hence, jack fruit seed at 8% level was used for optimization amylase production. Ikram-ul-Haq *et al*. (2003) used a low-cost fermentation for the production of α-amylase. Biesebeke *et al*. (2005) applied flours and cereal bran, potato residue and starchy waste, as the substrate for enzyme production by filamentous fungi. Rajagopalan and Krishnan (2010) used oil cakes for the production of α-amylase by *Bacillus subtilis* KCC103. Considering the enzyme yield, the jack fruit seed powder was used as a novel substrate for the production of amylase.

Among physio-chemical parameters in enzyme bioprocess, the pH of the culture medium played a critical role by inducing enzyme production. The optimum pH range for the production of amylases ranged between 6.0 and 7.0 (Liu *et al*., 2008). In this study, the optimum pH for amylase production in *Penicillium* sp. SP2 was 6.0 (Fig. 1b), which was similar to the findings of Geetha *et al*. (2011) who reported in *Aspergillus flavus*. Conversely, this might be due to the requirement of acidic pH for the growth and production of enzymes (Liu *et al*., 2008; Sun *et al*., 2009). The effect of temperature on amylase production was performed. Enzyme production was found to be maximum at 35 °C and declined therefter (Fig. 1c). This temperature optimum was higher than that of the reported enzyme production from *Penicillium* sp. (Gouda and Elbahloul, 2008). Various research groups have reported that 30 °C was found to be optimum temperature for the growth and enzyme production from fungal species (Shafique *et al*., 2009).

The fermentation process is affected by the initial inoculum concentration. There was a gradual increase in α-amylase production upto 9% inoculum level, and amylase production decreased above this optimum level (Fig. 2a). The results of the present study is in accordance with the observations made by *Bacillus cereus* MTCC 1305 in SSF (Anto *et al*., 2006). In *A. oryzae*, 5%–8% inoculum level was found to be optimum for the production of amylases. Irfan *et al*. (2012) reported that 10% inoculum level was found optimum for amylase production by *Rhizopus oligosporus*-ML10. The results shown in Fig. 2b indicated that amylase production was 8.23 U ml^{-1} after 7 days of incubation and decreased thereafter. After 7 days, amylase production decreased continuously, which may be due to denaturation of amylase. This study was similar to that of the reported study from *A. niger* strain VB6 (Joel and Bhimba, 2012).

Different carbon sources, including glucose, starch, lactose, sucrose and fructose were used for amylase production. Among all the carbon sources, glucose (9.90 ± 0.39) elicited the maximum production of amylase (Fig. 3a). All the other supplemented carbon sources also enhanced amylase production than control. For further studies, glucose was selected as the best carbon source. The result shown in Fig. 3b indicated that the yeast extract enhanced amylase production than other nitrogen supplements. All supplemented nitrogen sources enhanced enzyme production. The beneficial

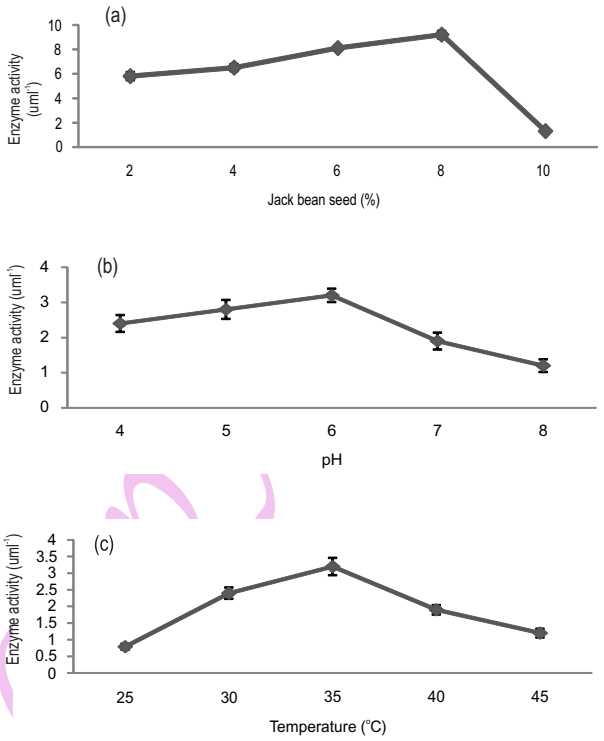


Fig. 1 : (a) Effect of different concentration of substrate on enzyme production; (b) Effect of pH on enzyme production and (c) Effect of temperature on enzyme production. Error bar: SD among replicates.

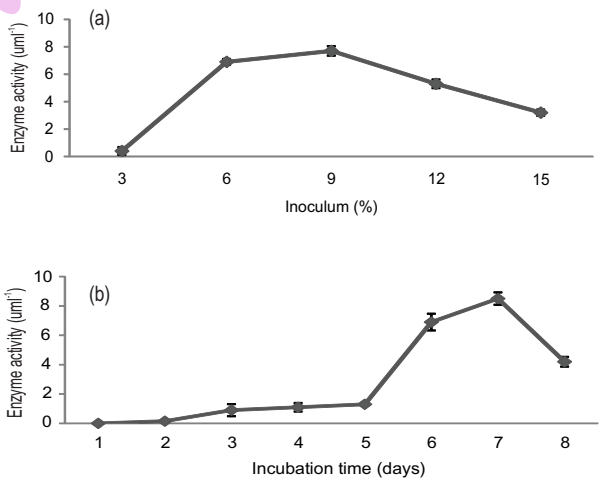


Fig. 2 : (a) Effect of inoculum on enzyme production and (b) Effect of incubation period on enzyme production. Error bar: SD among replicates

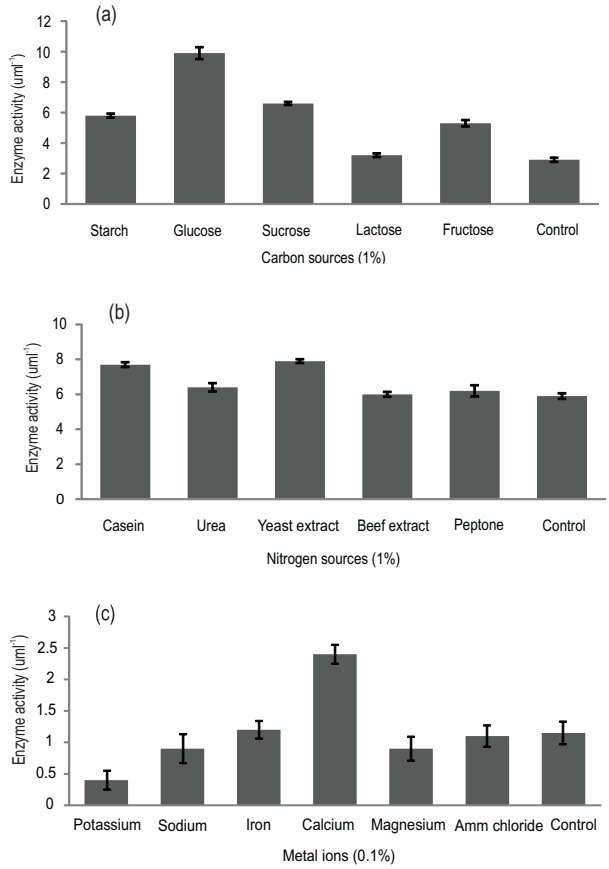


Fig. 3 : (a) Effect of carbon sources on enzyme production; (b) Effect of nitrogen sources on enzyme production and (c) Effect of metal ions on enzyme production. Error bar: SD among replicates

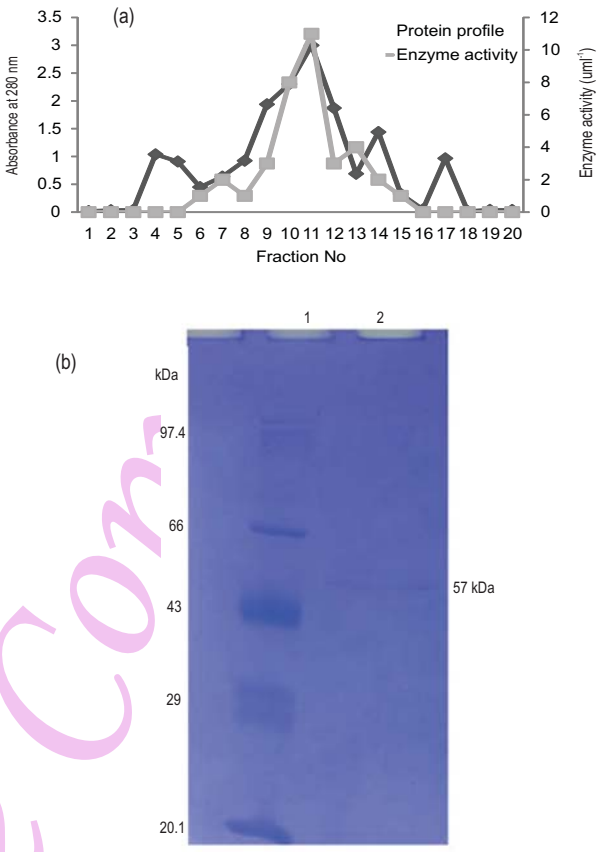


Fig. 4 : (a) Elution profile of amylase from sephadex G-75 gel filtration chromatography. and (b) Sodium dodecyl sulphate polyacrylamide gel electrophoresis (12%) of purified amylase from *Penicillium* sp. SP2 [Lane 1- protein marker; Lane 2 – purified enzyme]. The molecular weight of amylase was 57 kDa

effects of adding beef extract for amylase production has been reported earlier (Varalakshmi *et al*., 2009; Geetha *et al*., 2011). Previous findings have shown that casein hydrolysate, sodium nitrate and peptone were the best nitrogen supplements for amylase production in *Thermomyces lanuginosus* and *A. niger* (Kunamneni *et al*., 2005; Shivaramakrishnan *et al*., 2007). Supplementing mineral salts influence the growth of microorganism and thereby, stimulate enzyme production. In the present study, among the metal ions supplemented Ca^{2+} ion enhanced amylase production (Fig. 3c). This result is in accordance with the observations made earlier with other fungal species. Most of the fungi require metal ions for the production of amylases (Aiyer, 2004). In *B. subtilis*, supplementation of $CaCl_2$ effectively increased enzyme production in SSF (Rameshkumar and Sivasudha, 2011).

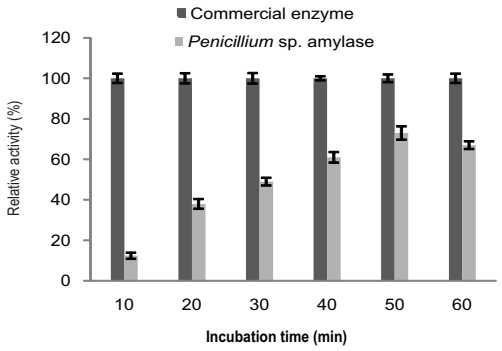


Fig. 5 : Effect of amylase from *Penicillium* sp. SP2 in wastewater treatment

Amylase was purified by ammonium sulphate precipitation and sephadex G - 75 gel filtration chromatography. The sephadex G-75 gel filtration column yielded one major peak and three minor peaks (Fig. 4a). The molecular weight of amylase was found to be 57 kDa (Fig. 4b). The molecular weight of amylase was reported to be 60.5 kDa in *Penicillium camemberti* PL21 (Nouadri *et al*., 2010). The molecular weight of amylase from *Penicillium* sp. NIOM-02 was reported to be 53 kDa (Dhale and Raj, 2009). These findings showed variation in molecular weight of amylase among *Penicillium* sp. The

molecular weight of amylase was 58 kDa in *Talaromyces pinophilus* (Xian *et al*., 2015). In wastewater, the pollutants decrease the level of DO. Several pharmaceutical, food and textile industry effluents are rich in fibre, starch and other organic pollutants which are toxic for the environment. In the present study, both commercial enzyme and amylases from *Penicillium* sp. SP2 increased the DO content in wastewater (Fig. 5). This result is in accordance with the observations made with amylase from *Aspergillus gracilis*. The wastewater contains effluents, ions, inhibitors, surfactants and other insoluble matters. Hence, the activities of amylase in wastewater treatment mainly depends on the biochemical properties of the enzyme (Ali *et al*., 2014). The increased DO content in amylase treated wastewater revealed that the enzyme breakdown organic matters in wastewater. When the incubation time increases, dissolved oxygen content of the wastewater increases. The results revealed that amylase from *Penicillium* sp. SP1 was effective to treat wastewater. Uwadiae *et al*. (2011) studied the effect of amylase to treat pharmaceutical effluent using amylase and reported increased DO after the application of amylase.

Penicillium sp. SP2 has potential to utilze jack fruit seed for the growth and production of amylase. Supplementation of carbon source (glucose), nitrogen source (beef extract), and Ca^{2+} ions enhanced amylase production in SSF. Considering its nutritive value and its cheap cost, jack fruit seeds can be useful for the production of amylase in industrial scale. This organism hydrolyzed carbohydrates and increased DO. Hence, this organism can be useful to treat sewage water.

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